Evidence Brief

Rationale

High grade serous carcinoma (HGSC) is the most common and most aggressive subtype of epithelial ovarian cancer (OC). The majority of patients present in the advanced stage with poor prognosis. If diagnosed in the earliest stage (stage I) survival is greatly improved; however, there is currently no effective screening method for OC. Strong pathological evidence supports the theory that the distal fallopian tube is the origin of HGSC and a precursor lesion known as serous tubal epithelial carcinoma (STIC) has been identified. DNA methylation (DNAme) aberrations occur as an early event in carcinogenesis. The use of minimally invasive blood sampling, or 'liquid biopsies' is a fast-emerging area of cancer diagnostics. Identifying and developing novel DNAme markers for the detection of HGSC in blood samples, will fulfil an unmet clinical need in a poor outcome cancer.

Methods

A short list of 20 differentially hypermethylated DNA regions were identified following DNAme array profiling (on the Illumina® Infinium 450K platform). Pyrosequencing analysis, probe-based quantitative PCR and digital PCR were employed to analyse a selection of candidate DNAme markers initially in a discovery cohort (6 patients with matched NFT-SIC-HGSC tissue samples) and subsequently in a validation cohort (48 NFT and 48 HGSC samples) of FFPE tissue samples. The most promising DNAme marker was analysed in a cohort of matched tissue and plasma samples using an optimised methylation-sensitive restriction enzyme PCR (MSRE qPCR) protocol.

Findings

Of the 20 differentially methylated regions (DMRs) analysed using pyrosequencing, seven showed statistically significant hypermethylation ($p<0.0001$) from NFT-STIC-HGSC FFPE tissue samples in the discovery cohort. These DMRs, termed DNAme markers, were taken forward for Pyrosequencing in the validation cohort, revealing statistically significant ($p<0.0001$) hypermethylation in HGSC for all seven DNAme markers. Probe-based quantitative Methylation-Specific PCR (MSP) was then performed (for use on a Roche Lightcycler) with four DNAme markers showing hypermethylation in HGSC tissue samples compared to NFT. Statistically significant hypermethylation was observed in Stage I disease compared to NFT in all four markers. A ROC analysis comparing these four markers with serum CA125 showed whilst they had comparable sensitivity, DNAme markers outperformed serum CA125 in terms of specificity. Given the low concentrations of DNA obtained following cell free DNA (cfDNA) extraction from plasma samples, an optimised MSRE qPCR protocol was developed for the most promising DNAme marker. Statistically significant hypermethylation ($p=0.009$) was detected using MSRE qPCR in plasma cfDNA for this marker. Area under curve (AUC) in the matched FFPE tissue and plasma samples was 0.9375 and 0.8646, respectively.

Recommendations

This study has provided a proof-of-principle for the identification of novel DNAme biomarker for HGSC. A unique biomarker discovery pipeline has been established and optimised to use in the development of potential DNAme blood-based biomarkers for HGSC.

Background

‘Ovarian’ cancer and HGSC: Ovarian cancer (OC) is an umbrella term for a multitude of different types of cancer that affect the ovaries, fallopian tubes and the primary peritoneal cavity. There is a wide variation in terms of incidence and outcomes for these different types. The majority (60–70%) are epithelial in origin; which includes high grade serous carcinoma (HGSC) (68%), clear cell (13%), endometrioid (9%) and mucinous (3%).[1,2] In the UK, OC is the sixth most common cancer and the most common cause of death from gynaecological malignancies.[3] Around 7,500 new OC cases are diagnosed in the UK every year, with incidence rates highest amongst women aged 75 to 79. Epithelial ovarian cancer (EOC) is the most common cause of death from gynaecological malignancy in the developed world, with most deaths being attributed to HGSC.[4] Almost 75% of women with OC present at a late stage (58% stage 3, 17% stage 4), with associated 5-year survival rates of approximately 35%.[3] Approximately 25% of women present with early stage disease (20% stage 1, 5% stage 2). If detected at an early stage survival rates increase up to 80-95%.[3]

HGSC originates in the fallopian tube: Historically, most theories of the pathophysiology of OC included the concept that it begins with the dedifferentiation of the cells overlying the ovary, the ovarian surface epithelium (OSE). For decades the incessant ovulation theory was the most accepted hypothesis of OC carcinogenesis.[5] One of the major advances in our understanding of the pathogenesis of OC was the recognition that a high proportion of HGSCs may originate from distal fallopian tube epithelium or the tuboperitoneal junction rather that the OSE.[6] Beginning with the discovery of the *BRCA*-associated ovarian cancer susceptibility genes and subsequent examination of risk-reducing salpingo-oophorectomy (RRSO) specimens, a new model of ovarian carcinogenesis began to unfold drawing attention to the distal fallopian tube as a more likely site of origin for HGSC.[7,8,9] HGSC is characterised by ubiquitous *TP53* mutation. The most compelling evidence for the proposed new site of origin came from a series of confirmatory reports which identified early serous cancers containing *TP53* mutations in the fallopian tube but not the ovary.[10,11,12,13] Subsequent studies identified the presence of potential precursor lesions in the distal fallopian tube in high-risk women.[14,15] Serous intraepithelial or early invasive carcinomas were found in up to 10% of fallopian tubes in *BRCA* mutation carriers who had undergone prophylactic bilateral salpingo-oophorectomies. These proliferations, termed serous tubal intraepithelial lesions (STIL) or serous tubal intraepithelial carcinomas (STIC), demonstrated identical *TP53* mutations to adjacent HGSC.[15,16] Following on from these initial studies, several studies have since reported the detection of STICs in up to 60% of women with HGSC, in both hereditary and sporadic disease.[17,18,19] Preliminary work from our laboratory has supported the evolutionary trajectory of HGSC origin from fallopian tube and identified additional oncogenic drivers of HGSC carcinogenesis.[20]

‘Ovarian’ cancer screening: Cancer screening has been shown to improve mortality rates in cancers such as breast, cervical, prostate and colorectal cancer. Unlike the successful screening programmes that have been developed for these cancers, there is currently no acceptable programme for OC. This is in part due to the invasive nature of obtaining tissue samples from patients with suspected OC and, until recently, a lack of identifiable precancerous lesions. Furthermore, as OC has a relatively low prevalence rate, screening strategies require a high sensitivity (>75%) and specificity (99.6%) with a positive predictive value (PPV) of at least 10%.[21] Multiple efforts have been made to improve survival rates through early screening methods based on serum CA125 levels and TVUS.[22,23] Thus far, none of these methods have met the standards required to advocate population-based screening.

Liquid biopsy: Precision oncology seeks to obtain molecular information about cancer to improve patient outcomes. Tissue biopsy samples are widely used to characterise tumours; however, this method of tumour analysis is limited by constraints on sampling frequency and incomplete representation of the entire tumour. The term ‘liquid biopsy’ was first used to describe methods that can derive the same diagnostic information from a blood sample, or other body fluids, that is typically

derived from a tissue biopsy sample.[24] In recent years, the focus of precision medicine is increasingly turning towards liquid biopsies as they are non-invasive and can be repeated at multiple time points facilitating 'real-time' disease monitoring.[25] Liquid biopsy can include measurement of soluble factors, such as circulating tumour nucleic acids (DNA/RNA), circulating tumour cells (CTCs), proteins, and extracellular vesicles such as exosomes. The presence of fragments of circulating cfDNA in blood was first described in 1948.[26] Several decades later, the clinical importance of this finding was recognised when researchers observed higher levels of cfDNA in the serum of cancer patients compared to healthy individuals.[27] Further studies demonstrated that cancer cells release cfDNA fragments into the circulation and other bodily fluids (referred to as ctDNA) and these fragments carry all the genetic and epigenetic characteristics of the primary tumour.[28,29] These proof-of-principle studies lead to a surge in research into the potential applications of cfDNA in cancer management. As a result, cfDNA analysis now has multiple indications in oncology including early detection, diagnosis, staging and prognosis, monitoring response to treatment, monitoring minimal residual disease and relapse and identifying acquired drug resistance mechanisms.

DNA methylation as a biomarker: Increasing evidence has shown that epigenetic alterations including DNAme play an important role in cancer, from silencing of tumour suppressor genes (TSG) to activation of oncogenes and the promotion of metastasis.[30] Herman *et al.* were among the first to show that DNA hypermethylation is directly involved in carcinogenesis.[31] Since then, there has been growing appreciation of its role in early cancer development and progression.[32] The molecular, clinical and pathological features of OC have been associated with distinct DNAme patterns. Some recent studies have focused on genome-wide identification of methylated biomarkers in OCs. The methylated DNA immunoprecipitation microarray (MeDIP-chip) identified 367 CpG islands specifically methylated in OC compared to normal ovarian tissue.[33] The TCGA surveyed 519 HGSC tumours using the Illumina Human Methylation 27K BeadChip array and found 168 genes exhibiting aberrant DNA hypermethylation with associated reduced gene expression.[34] Surprisingly, despite minimal blood-borne spread, aberrant DNAme can be detected in serum, plasma and peritoneal fluid of OC patients.[35] DNAme has several advantages compared to other molecular biomarkers. Methylation analysis utilises DNA which is chemically more stable than other molecules, such as RNA and protein. DNAme patterns are also chemically and biologically stable and are relatively unaffected by physiological state and sample collection conditions.[36] Furthermore, after acquiring a methylation alteration, the methylation pattern is generally conserved throughout disease progression. Compared to genetic mutations, DNAme patterns are easier to detect as they are binary signals (methylated or unmethylated) tend to occur in specific regions (CGIs) and can be easily amplified using PCR.[37] In contrast, genetic alterations may vary considerably from patient to patient, even within the same cancer type, and can be spread over large sections of DNA, necessitating the need for more complex analytic tools. Altered DNAme patterns have been associated with early cancer development, sensitivity to treatment and the process of metastasis.[38,39,40] A number of studies have shown the feasibility of detecting altered methylation patterns in circulating DNA in a broad range of cancers, including OC.[41,42,43] Thus, tumour specific methylation in cfDNA is an appealing target for the development of non-invasive, blood-based assays for cancer diagnosis.

Aims and Objectives

Hypothesis:

DNA methylation events present in early disease (STIC) should be detectable in cell free DNA extracted from plasma samples of HGSC patients and could represent the basis of a more sensitive and specific, diagnostic test than the current marker, CA125.

Overall Objective:

The development of early detection DNA methylation blood-based biomarkers for HGSC of the fallopian tube/ovary.

Specific Aims:

1. To utilize our new knowledge of the origin of HGSC to identify genomic regions that are differentially methylated between NFT-STIC-HGSC

2. To investigate the potential use of methylated cfDNA in blood as early warning diagnostic biomarkers

Methods

Previous work within our group identified the candidate DMRs that have been taken forward for validation in this study.[20] In the previous study, DNAme profiling was carried out using the Illumina® Infinium Human Methylation 450K BeadChip® platform on the same pilot cohort analysed in this study. An analysis of differential methylation was conducted according to the following comparisons: 1) NFT-HGSC, 2) NFT-STIC and 3) STIC-HGSC. β values reported by the 450K Illumina® platform for each probe represent the methylation level measurement for a targeted CpG site. The range of the β value is from 0 (no methylation) to 1 (100% methylation). A higher β value indicates a higher DNAme level. Differential methylation was computed based on the difference in mean β values (methylation levels) of the two groups being compared. A shortlist of 20 candidate DMRs for this study were identified based on the top ranking differentially hypermethylated CpG sites within the NFT-HGSC comparison, as determined in the previous study.

Pyrosequencing analysis, probe-based methylation specific quantitative PCR and digital PCR were employed to analysis a selection of candidate DNAme markers initially in the discovery cohort (6 patients with matched NFT-SIC-HGSC tissue samples) and subsequently in a validation cohort (48 NFT and 48 HGSC samples) of FFPE tissue samples. The most promising DNAme marker was analysed in a cohort of matched tissue and plasma samples (n=16) using an optimised methylation-sensitive restriction enzyme PCR (MSRE qPCR) protocol. The same assay was used to evaluate the dynamic changes of this DNAme marker in a longitudinal cohort of eight patients from the time of cytoreductive surgery to completion of standard chemotherapy.

Personal and Public Involvement

The Northern Ireland Cancer Research Consumer's Forum were contacted during the development of this project and the proposal was received with great enthusiasm. Feedback from the NICRCF committee members was taken into consideration in the finalised proposal. This Forum is a great source of patient and public involvement and allows communication between patients, their families, members of the general public and clinicians in a non-clinical environment. Throughout the duration of this project the NICRCF were consulted and kept up to date on the progress of this project through attendance at NICRCF meetings and annual presentation of project progression and results.

The BRCA-Link NI patient support group (www.brcani.co.uk) was established in Belfast in 2010. This group is now well recognised by BRCA-linked families and clinicians as the main BRCA patient forum in NI. The forum provides a focus group for topical debate and provides support for BRCA-positive women who have suffered or who are at risk of developing breast and/or ovarian cancer. BRCA-Link NI have close ties with CCRCB-QUB and recent joint meetings and patient information days have been very useful in ascertaining the public perception of ovarian cancer screening and "early" diagnosis. This cohort of patients were not fully aware of the lack of an adequate screening test, not to mention an accurate disease-specific blood test. They were in favour of further research to establish a highly sensitive and specific ovarian cancer diagnostic biomarker. The results of this project were presented at the annual BRCA-Link NI meeting in February 2019.

The ongoing communication with both of these forums facilitated a partnership between the general public and the research team at CCRCB which undoubtedly improved the nature of this translational study.

Findings

Of the 20 regions analysed, 8 were identified as consistently progressively hypermethylated from NFT-STIC-HGSC in this six-patient cohort (4 DNAme markers shown in Figure 1). These markers were taken forward for pyrosequencing in a validation cohort of 48 NFT and 48 HGSC (unmatched) FFPE tissue samples, revealing statistically significant ($p<0.0001$) hypermethylation in HGSC for all 4 DNAme markers (Figure 2). Probe-based quantitative Methylation-Specific PCR (MSP) was then performed (for use on a Roche Lightcycler) with all 4 DNAme markers showing statistically significant hypermethylation (Figure 3). When these tumours were stratified into FIGO stages we saw that all 4 markers were significantly hypermethylated, even in Stage 1 disease and maintained at high levels through later stages (Figure 4). A ROC analysis comparing these 4 markers with serum CA-125 showed whilst they had comparable sensitivity, DNAme markers outperformed serum CA-125 in terms of specificity (Figure 5).

We then employed quantitative Methylation Specific Restriction Endonuclease PCR (MSRE PCR) – a technique based on the inability of specific restriction endonucleases to cleave methylated DNA. A major advantage of MSRE PCR is that bisulphite conversion is not required, thus minimising DNA degradation. Figure 6 shows MSRE-PCR for the first two markers, CG1 and CG12, both significantly hypermethylated relative to NFT and importantly, hypermethylated in early disease.

Logistical regression (XGBoost) was performed on pyrosequencing data to determine which of the first 4 markers possessed the greatest diagnostic potential. As Figure 7 shows, CG1 performed best but the combination all 4 markers predicted 8 out of 9 HGSC samples correctly, when applied to independent methylation data.

We could detect CG1 hypermethylation by MSRE PCR, with as little as 5 ng plasma cfDNA and we optimised this for use in Droplet Digital PCR, DD-PCR (Figures 8A and 8B). Droplet Digital MSRE PCR of CG1 (using 48 NFT v 48 HGSC samples) again showed highly significant HGSC hypermethylation (Figure 8C). Using longitudinal samples, the CG1 MSRE-PCR signal showed signs of elevation in some samples post cytoreductive surgery (Figure 9A), whilst matched CA125 assays continued to fall during chemotherapy cycles (Figure 9B).

Fig1

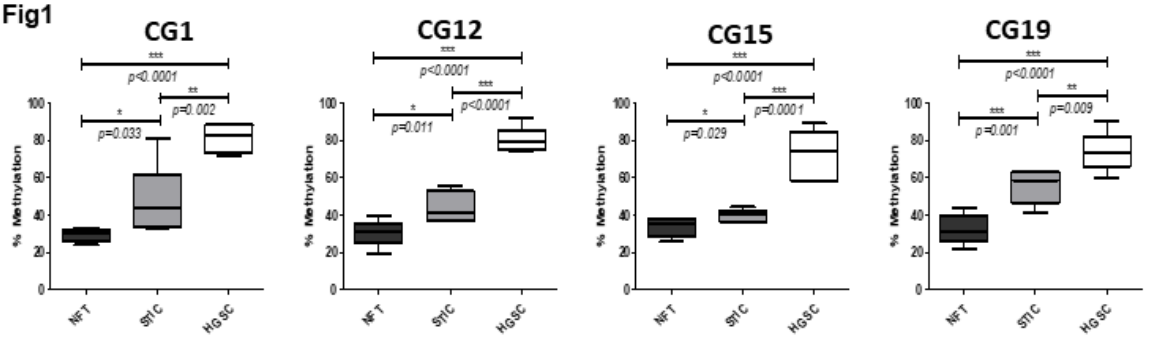


Fig1. Bar graphs showing pyrosequencing values for 4 DNAme marker regions in the discovery cohort of 6 Normal Fallopian tube (NFT), 6 Serous Tubal Intraepithelial Carcinoma (STIC) and 6 High Grade Serous Carcinomas (HGSC). *p<0.05, **p<0.01, ***p<0.0001.

Fig2

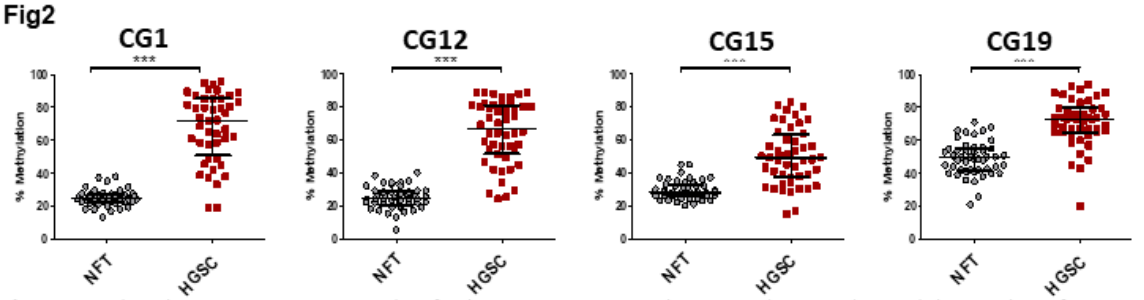


Fig2. Scatterplots showing pyrosequencing values for the same 4 DNAme marker regions (as in Fig1) in a validation cohort of 48 Normal Fallopian Tube (NFT) and 48 High Grade Serous Carcinomas (HGSC). ***p<0.0001.

Fig3

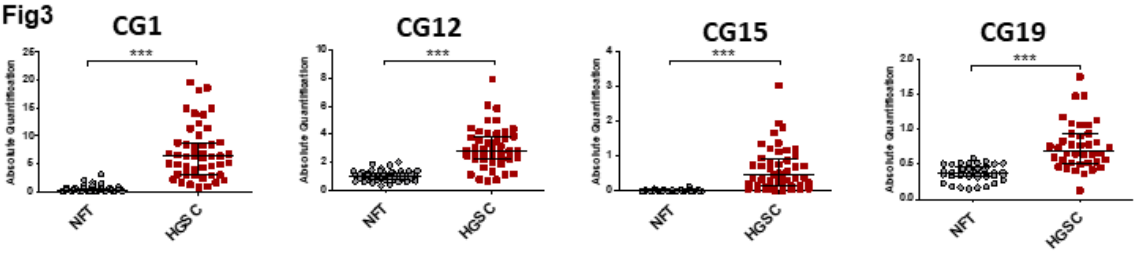


Fig3. Scatterplots showing Methylation-Specific PCR values for the same 4 DNAme marker regions (as in Fig1) in a validation cohort of 48 Normal Fallopian Tube (NFT) and 48 High Grade Serous Carcinomas (HGSC). ***p<0.0001.

Fig4

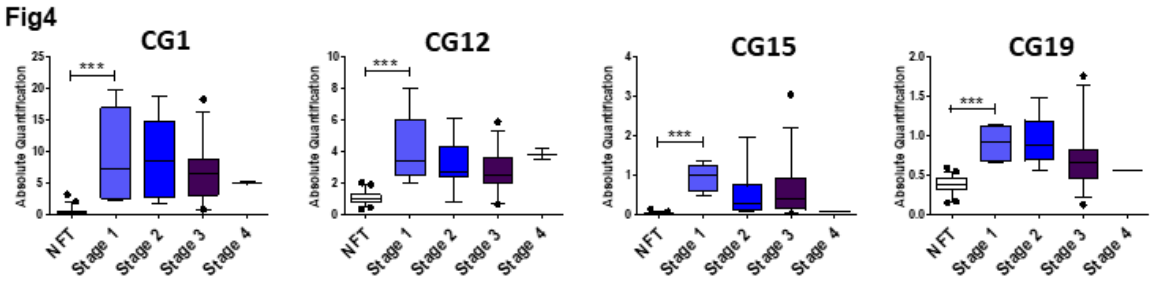
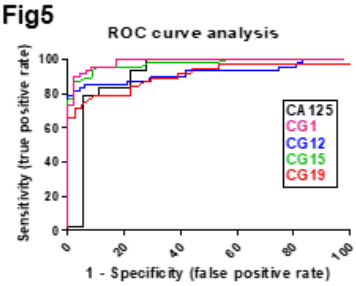


Fig4. Bar graphs showing the same Methylation-Specific PCR values shown in Fig3, this time with HGSCs stratified by FIGO stage. This comparison shows that all 4 markers are elevated relative to NFT in early disease. *p<0.05, **p<0.01, ***p<0.0001.

Fig5



	CA125 (n=66)	CG1 (n=95)	CG12 (n=95)	CG15 (n=93)	CG19 (n=79)
Sensitivity (95%CI)	**97.92%** (88.93-99.95)	**91.67%** (80.02-97.68)	**83.33%** (69.78-92.52)	**87.23%** (74.26-95.17)	**66.67%** (51.59-79.60)
Specificity (95%CI)	**72.22%** (46.52-90.31)	**95.74%** (85.46-99.48)	**93.62%** (82.46-98.66)	**97.83%** (88.47-99.94)	**97.92%** (88.93-99.95)
AUC (95%CI)	**0.912** (0.805-1.019)	**0.985** (0.969-1.002)	**0.919** (0.858-0.981)	**0.973** (0.945-1.002)	**0.893** (0.825-0.962)

Fig5. ROC analysis showing increased specificity of all 4 DNAme markers, relative to CA125.

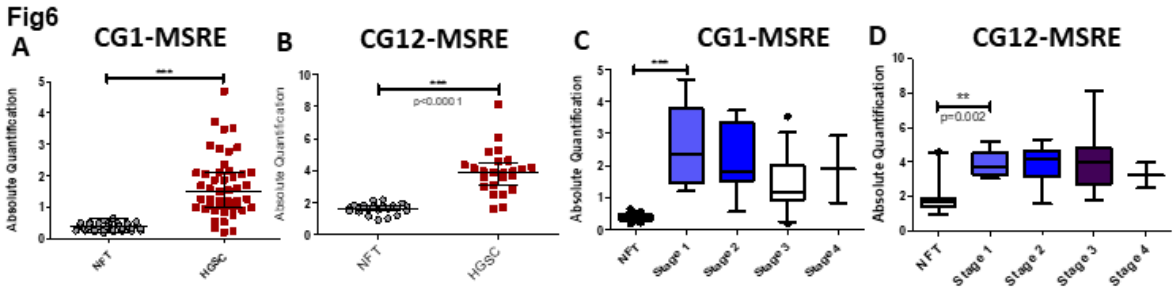


Fig6. Scatterplots (A, B) showing Methylation-Specific Restriction Enzyme PCR (MSRE-PCR) values for CG1 and CG12 in the validation cohort of 48 Normal Fallopian Tube (NFT) and 48 High Grade Serous Carcinomas (HGSC). Bar graphs (C, D) show breakdown by FIGO stage. ***p<0.0001.

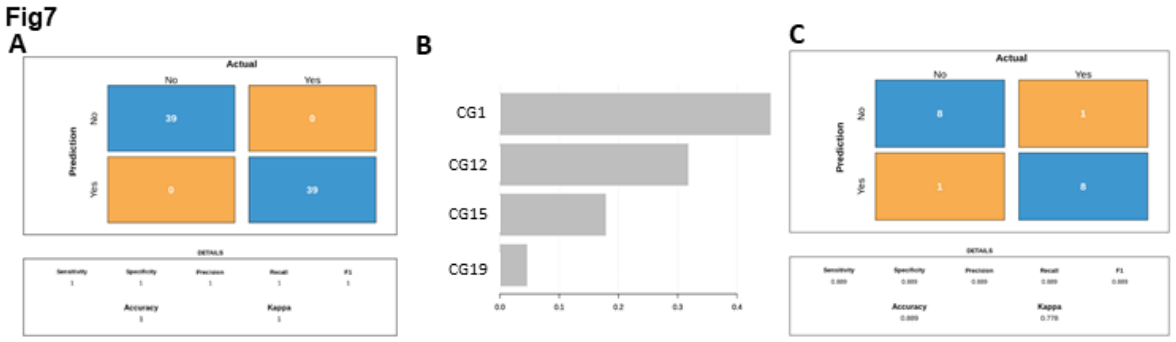


Fig7. Preliminary data from XG Boost logistical regression analyses. **A.** Training set of 39 samples using the 4 markers (shown in **B**) gave a correct call in 8 out of 9 HGSC cases (**C**). Note – as shown in **B**, CG1 was the strongest marker in this 4 set panel.

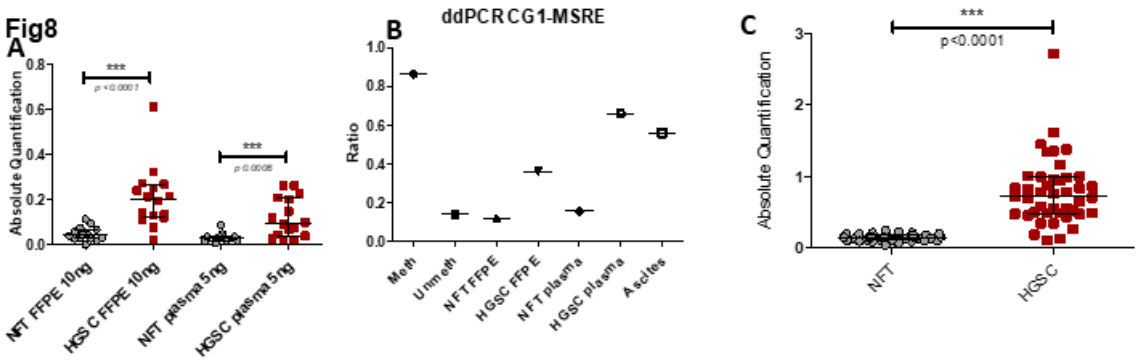


Fig8. Scatterplot showing CG1 MSRE-PCR values for 20 FFPE samples from NFT and HGSC (10ng DNA) compared to 20 matched plasmas (5ng DNA), showing that methlation of CG1 is significantly elevated and can be detected from only 5ng of plasma cfDNA. B. Optimisation of CG1 MSRE-PCR using DDPCR, which again shows that CG1 is elevated > 5-fold in plasma DNA from HGSC patient versus a non-cancer plasma, with ascites showing similar elevation. C. CG1 MSRE-PCR measured by DDPCR in 48 NFT v 48 HGSC cohort. ***p<0.0001.

Fig9A CG1 MSRE PCR on plasma

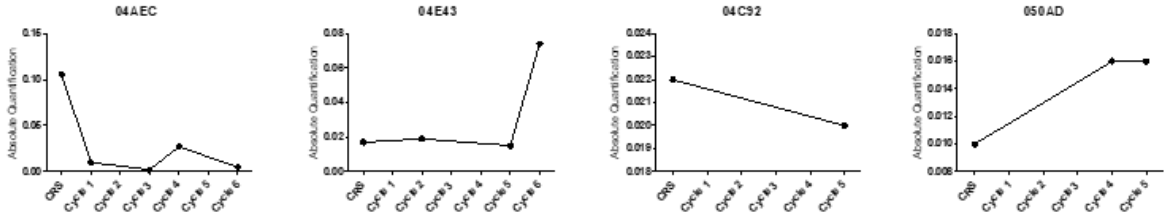
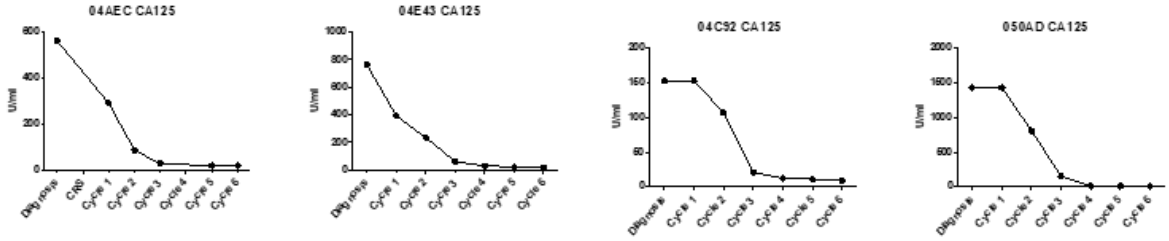


Fig9B Matched plasma samples – CA125 measurements



Conclusions and Recommendations

The initial discovery phase of the candidate DNAme biomarkers identification for this study was implemented using matched tissue samples from each stage of the carcinogenic pathway of HGSC: NFT, STIC and HGSC.[350] Although the sample size was small it provided a highly unique sample set from which to identify potential early detection HGSC-specific DNAme biomarkers. The identification of DNAme markers showing hypermethylation in STIC lesions was also an essential component in choosing potential HGSC-specific early detection biomarkers.

The current study served to develop assays for the most promising HGSC-specific DNAme markers and evaluate their potential as blood-based biomarkers. Eight candidate DNAme markers were developed and extensively optimised by Pyrosequencing analysis. The diagnostic accuracy of two DNAme markers (CG1 and CG12) surpassed that of the gold standard, CA125, in tissue samples. MSP assays were developed and evaluated for 4 DNAme markers, two of which (CG1 and CG12) again showed improved accuracy in detecting HGSC compared to CA125. The bisulphite conversion step required for MSP analysis posed a number of challenges, namely in degrading DNA quality and yield, and was therefore abandoned in favour of a technique that would avoid the need for bisulphite conversion, MSRE PCR. Prohibitively low cfDNA yields obtained from the patient plasma samples lead to the development of a targeted MSRE pre-amplification strategy, with subsequent proof-of-concept validation of one marker, CG1, in a small matched tissue and plasma cohort. The CG1 DNAme marker distinguished the HGSC group from the NFT group using cfDNA extracted from plasma samples. The marker outperformed the current gold standard, CA125, in terms of specificity (CG1, specificity 86.67%; CA125, specificity 72.22%); however, the overall diagnostic accuracy of CA125 was higher (CA125 AUC 0.912; CG1 AUC 0.8646). As eluded to previously, it is unlikely that any single DNAme marker will possess the desired sensitivity and specificity required to accurately diagnose a heterogeneous disease such as HGSC, but a combination of such markers could have real potential as a diagnostic tool, either complementing or superseding CA125.

The final objective of this study was to evaluate the dynamic changes of the successfully developed MSRE qPCR assay in a prospective longitudinal blood collection cohort. Due to limited sample size and limited follow up time, definitive conclusions regarding the dynamics of this marker could not be

drawn at this stage. The low magnitude of methylation detected in the plasma cohort compared to the tissue cohort raised concerns that the sensitivity of the MSRE qPCR assay may not be adequate. The capacity to measure DNA methylation is improving in sensitivity. Droplet Digital PCR (ddPCR) amplifies and detects a single target sequence in a nanolitre reaction volume, helping to reduce competition from the non-specific product.[387] For this reason, the same assay was brought forward for development as a ddPCR-based MSRE assay. The ddPCR assay is still under development and further optimisation is ongoing as part of the researchers Academic Clinical Lecturer position.

This study has served to provide an exciting proof-of-principle pipeline of discovery and assay development for future potential HGSC-specific DNAme blood-based biomarkers. It is clearly evident that earlier and more accurate diagnosis of HGSC would have significant impact on the overall survival of this very poor outcome disease.

References

1. Soslow RA. Histologic subtypes of ovarian carcinoma: An overview. *Int J Gynecol Pathol*. 2008;27(2):161-174. doi:10.1097/PGP.0b013e31815ea812
2. McCluggage WG. Morphological subtypes of ovarian carcinoma: A review with emphasis on new developments and pathogenesis. *Pathology*. 2011;43(5):420-432. doi:10.1097/PAT.0b013e328348a6e7
3. Cancer Research UK www.cancerresearchuk.org/cancer-info/cancerstats/types/ovary/incidence/uk-ovarian-cancer-incidence-statistics: Accessed August 2019.
4. Delair D, Soslow RA. Key features of extrauterine pelvic serous tumours (fallopian tube, ovary, and peritoneum). *Histopathology*. 2012;61(3):329-339. doi:10.1111/j.1365-2559.2011.04167.x
5. Fathalla MF. Incessant ovulation – a factor in ovarian neoplasia. *Lancet*. 1971;298(7716):163. doi:10.1016/S0140-6736(71)92335-X
6. Kurman RJ, Shih IM. The dualistic model of ovarian carcinogenesis revisited, revised, and expanded. *Am J Pathol*. 2016;186(4):733-747. doi:10.1016/j.ajpath.2015.11.011
7. Friedman LS, Ostermeyer EA, Szabo CI, et al. Confirmation of BRCA1 by analysis of germline mutations linked to breast and ovarian cancer in ten families. *Nat Genet*. 1994;8(4):399-404. doi:10.1038/ng1294-399
8. Zweemer RP, Van Diest PJ, Verheijen RHM, et al. Molecular evidence linking primary cancer of the fallopian tube to BRCA1 germline mutations. *Gynecol Oncol*. 2000;76(1):45-50. doi:10.1006/gyno.1999.5623
9. Beirne JP, McArt DG, Roddy A, et al. Defining the molecular evolution of extrauterine high grade serous carcinoma. *Gynecol Oncol*. September 2019. doi:10.1016/j.ygyno.2019.08.029
10. Piek JMJ, Van Diest PJ, Zweemer RP, et al. Dysplastic changes in prophylactically removed Fallopian tubes of women predisposed to developing ovarian cancer. *J Pathol*. 2001;195(4):451-456. doi:10.1002/path.1000

11. Piek JM, van Diest PJ, Zweemer RP, Kenemans P, Verheijen RH. Tubal ligation and risk of ovarian cancer. *Lancet*. 2001;358(9284):844. doi:10.1016/S0140-6736(01)05992-X
12. Piek JMJ, Verheijen RHM, Kenemans P, Massuger LF, Bulten H, Van Diest PJ. BRCA1/2-related ovarian cancers are of tubal origin: A hypothesis [1]. *Gynecol Oncol*. 2003;90(2):491. doi:10.1016/S0090-8258(03)00365-2
13. Finch A, Beiner M, Lubinski J, et al. Salpingo-oophorectomy and the risk of ovarian, fallopian tube, and peritoneal cancers in women with a BRCA1 or BRCA2 Mutation. *JAMA*. 2006;296(2):185-192. doi:10.1001/jama.296.2.185
14. Lee Y, Miron A, Drapkin R, et al. A candidate precursor to serous carcinoma that originates in the distal fallopian tube. *J Pathol*. 2007;211(1):26-35. doi:10.1002/path.2091
15. Jarboe EA, S Pizer E, Miron A, Monte N, Mutter GL, Crum CP. Evidence for a latent precursor (p53 signature) that may precede serous endometrial intraepithelial carcinoma. *Mod Pathol*. 2009;22(3):345-350. doi:10.1038/modpathol.2008.197
16. Crum CP, Drapkin R, Miron A, et al. The distal fallopian tube: A new model for pelvic serous carcinogenesis. *Curr Opin Obstet Gynecol*. 2007;19(1):3-9. doi:10.1097/GCO.0b013e328011a21f
17. Kindelberger DW, Lee Y, Miron A, et al. Intraepithelial Carcinoma of the Fimbria and Pelvic Serous Carcinoma: Evidence for a Causal Relationship. *Am J Surg Pathol*. 2007;31(2):161-169. doi:10.1097/01.pas.0000213335.40358.47
18. Przybycin CG, Kurman RJ, Ronnett BM, Shih IM, Vang R. Are all pelvic (Nonuterine) serous carcinomas of tubal origin? *Am J Surg Pathol*. 2010;34(10):1407-1416. doi:10.1097/PAS.0b013e3181ef7b16
19. Kuhn E, Kurman RJ, Vang R, et al. TP53 mutations in serous tubal intraepithelial carcinoma and concurrent pelvic high-grade serous carcinoma-evidence supporting the clonal relationship of the two lesions. *J Pathol*. 2012;226(3):421-426. doi:10.1002/path.3023
20. Beirne J. The identification and characterisation of disease-specific biomarkers in pelvic high grade serous carcinomas. 2016.
21. Bast RC. Early detection of ovarian cancer: new technologies in pursuit of a disease that is neither common nor rare. *Trans Am Clin Climatol Assoc*. 2004;115. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2263776/pdf/tacca00001-0288.pdf. Accessed September 11, 2019.
22. Cramer DW, Bast RC, Berg CD, et al. Ovarian cancer biomarker performance in prostate, lung, colorectal, and ovarian cancer screening trial specimens. *Cancer Prev Res (Phila)*. 2011;4(3):365-374. doi:10.1158/1940-6207.CAPR-10-0195
23. Jacobs IJ, Menon U, Ryan A, et al. Ovarian cancer screening and mortality in the UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS): a randomised controlled trial. *Lancet (London, England)*. 2016;387(10022):945-956. doi:10.1016/S0140-6736(15)01224-6

24. Pantel K, Alix-Panabières C. Circulating tumour cells in cancer patients: Challenges and perspectives. *Trends Mol Med*. 2010;16(9):398-406. doi:10.1016/j.molmed.2010.07.001
25. Crowley E, Di Nicolantonio F, Loupakis F, Bardelli A. Liquid biopsy: monitoring cancer-genetics in the blood. *Nat Rev Clin Oncol*. 2013;10(8):472-484. doi:10.1038/nrclinonc.2013.110
26. Mandel P, Metais P. *C R Seances Soc Biol Fil*. 1948;142(3-4):241-243. http://www.ncbi.nlm.nih.gov/pubmed/18875018. Accessed September 20, 2019.
27. Leon SA, Shapiro B, Sklaroff DM, Yaros MJ. Free DNA in the serum of cancer patients and the effect of therapy. *Cancer Res*. 1977;37(3):646-650. http://www.ncbi.nlm.nih.gov/pubmed/837366. Accessed September 20, 2019.
28. Stroun M, Anker P, Maurice P, Lyautey J, Lederrey C, Beljanski M. Neoplastic characteristics of the DNA found in the plasma of cancer patients. *Oncology*. 1989;46(5):318-322. doi:10.1159/000226740
29. M Wan JC, Massie C, Garcia-Corbacho J, et al. Liquid biopsies come of age: towards implementation of circulating tumour DNA. *Nat Rev Cancer*. 2017. doi:10.1038/nrc.2017.7
30. Wilting RH, Dannenberg JH. Epigenetic mechanisms in tumorigenesis, tumor cell heterogeneity and drug resistance. *Drug Resist Updat*. 2012;15(1-2):21-38. doi:10.1016/j.drup.2012.01.008
31. Herman JG, Baylin SB. Gene Silencing in Cancer in Association with Promoter Hypermethylation. *N Engl J Med*. 2003;349(21):2042-2054. doi:10.1056/NEJMra023075
32. Esteller M. Molecular origins of cancer: Epigenetics in cancer. *N Engl J Med*. 2008;358(11):1148-1159. doi:10.1056/NEJMra072067
33. Michaelson-Cohen R, Keshet I, Straussman R, Hecht M, Cedar H, Beller U. Genome-wide de novo methylation in epithelial ovarian cancer. *Int J Gynecol Cancer*. 2011;21(2):269-279. doi:10.1097/IGC.0b013e31820e5cda
34. Bell D, Berchuck A, Birrer M, et al. Integrated genomic analyses of ovarian carcinoma. *Nature*. 2011;474(7353):609-615. doi:10.1038/nature10166
35. Fleischhacker M, Schmidt B. Circulating nucleic acids (CNAs) and cancer-A survey. *Biochim Biophys Acta - Rev Cancer*. 2007;1775(1):181-232. doi:10.1016/j.bbcan.2006.10.001
36. Shapiro LJ, Mohandas T. DNA methylation and the control of gene expression on the human X chromosome. *Cold Spring Harb Symp Quant Biol*. 1983;47 Pt 2:631-637. doi:10.1101/sqb.1983.047.01.074
37. Baylin SB, Jones PA. A decade of exploring the cancer epigenome-biological and translational implications. 2011. doi:10.1038/nrc3130
38. Barton CA, Hacker NF, Clark SJ, O 'brien PM, O'Brien PM. No Title. *Gynecol Oncol*. 2008;109(1):129-139. doi:10.1016/j.ygyno.2007.12.017
39. Flanagan JM, Wilhelm-Benartzi CS, Metcalf M, Kaye SB, Brown R. Association of somatic DNA methylation variability with progression-free survival and toxicity in ovarian cancer patients. *Ann Oncol*. 2013;24(11):2813-2818. doi:10.1093/annonc/mdt370

40. Braicu EI, Paul J, Hergovich A, et al. Platinum-Based Chemotherapy Induces Methylation Changes in Blood DNA Associated with Overall Survival in Patients with Ovarian Cancer. *Clin Cancer Res*. 2016;23(9):2213-2222. doi:10.1158/1078-0432.ccr-16-1754
41. Hoque MO, Feng Q, Toure P, et al. Detection of aberrant methylation of four genes in plasma DNA for the detection of breast cancer. *J Clin Oncol*. 2006;24(26):4262-4269. doi:10.1200/JCO.2005.01.3516
42. Hoque MO, Begum S, Topaloglu O, et al. Quantitative detection of promoter hypermethylation of multiple genes in the tumor, urine, and serum DNA of patients with renal cancer. *Cancer Res*. 2004;64(15):5511-5517. doi:10.1158/0008-5472.CAN-04-0799
43. Leung WK, To K-F, Man EPS, et al. Quantitative Detection of Promoter Hypermethylation in Multiple Genes in the Serum of Patients with Colorectal Cancer. *Am J Gastroenterol*. 2005;100(10):2274-2279. doi:10.1111/j.1572-0241.2005.50412.x